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Genomics Tools Available For Unravelling Mechanisms Underlying Agronomical Traits in Strawberry With More To Come

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Abstract body text

In the last few years, high-throughput genomics promised to bridge the gap between plant physiology and plant sciences. In addition, high-throughput genotyping technologies facilitate marker-based selection for better performing genotypes. In strawberry, *Fragaria vesca* was the first reference sequence obtained in the Rosoideae sub-family. This genome has a high level of synteny with genomes of other species of diploid and polyploid *Fragaria*, but it also provides a reference for species like *Rubus* and *Rosa* for functional genomics.
Many tools for genetic, genomic and functional analyses were introduced over the last 10 years and these tools are still evolving. For genotyping, many studies have used simple sequence repeats (SSRs) but whole genome sequencing is now a mature technology and facilitates the development of genotyping chips and other genetic approaches such as genome wide association studies (GWAS). Furthermore, microarray-based technologies have been eclipsed by RNA-seq, the high-throughput sequencing of RNA. These new approaches have led to advances in our understanding of the genetically complex octoploid species, and have revolutionized functional genomics.
For all genetic and genomic studies, novel material such as complex crosses such as NILs and EMS have appeared in addition to the classical segregating population.

With all these tools, strawberry now emerges as a plant model, not only for studying fruit quality but also for deciphering the mechanisms controlling various aspects of plant biology.
Selective examples will be described to illustrate the latest research on strawberry and what is coming from other model species.

Keywords

omics, polyploidy, *Fragaria*, functional genomics

Introduction

In the last few years, high-throughput genomics promised to bridge the gap between plant physiology and crop sciences, and between classical breeding and marker assisted selection. The potential of genomics needs to be exploited for accelerated development of improved cultivars possessing high yield, genetic resilience against stresses, and enhanced nutritional quality. By using advanced breeding approaches, strawberry genetic enhancement programs will be intensified. In this perspective, the next- generation sequencing (NGS) and genotyping technologies need to be used for comparative genomics, marker-trait association, gene characterisation, and functional marker development, and these technologies must be deployed in routine breeding programs.
The objective of this review is to give an overview of genetics and genomics resources and development currently available for strawberry. We will also focus on genomics approaches for improving agronomical traits.

Sequencing and Re-Sequencing Efforts in *Fragaria*

The first published sequence was based on the perpetual flowering genotype Hawaii-4. Using solely Illumina-based sequencing, version 1.0 of this reference genome was released at the end of 2010 (Shulaev et al., 2011). Then, based on a denser microsatellite marker-based linkage map, some scaffolds were discarded to optimize the order of the linkage map and a new version, v1.1, was released. More recently, Tennessen et al., (2014) revised the assembly of v. 1.1 of the *F. vesca* reference assembly using a very dense linkage map from two crosses (one a selfed individual) of *F. vesca ssp. bracteata* which resulted in v. Fvb2.0.a1. When compared to the v1.1, Fvb2.0.a1 minimized genome rearrangements between *Prunus* and *Fragaria*. In addition to *F. vesca*, sequences of other diploid *Fragaria* species are available at the Genome Database for the Rosaceae (GDR) website (Jung et al., 2014).
The genome of *F. vesca* has a high level of macrosynteny with genomes of other species of diploid and polyploid *Fragaria*, (Rousseau-Gueutin et al., 2008; Sargent et al., 2009; Isobe et al., 2013; Sánchez-Sevilla et al., 2015) and other Rosaceae genomes like that of black raspberry, *Rubus occidentalis* (VanBuren et al., 2016). It also serves as a reference for species like *Rubus* and *Rosa* for functional genomics (Koning-Boucoiran et al., 2015).
In the cultivated octoploid strawberry, shotgun sequencing obtained 211,588 scaffolds that are not assigned to subgenomes (Hirakawa et al., 2014). These sequences, which cover 70.3% of the total length of the *F. vesca* genome (v1.1), highlight the high level of macrosynteny within *Fragaria* genomes.

From RAPD to Next Generation Genotyping and Sequencing Technologies

In the last decade, the repertoire of molecular markers was extensively improved and enlarged. Considerable efforts were undertaken to improve density of linkage maps in the octoploid strawberry and to optimize the choice of markers for fingerprinting sets. Markers that do not require sequence information were first developed and include Random Amplified Polymorphic DNA (RAPDs) (Degani et al., 2001, Sugimoto et al., 2005), amplified fragment length polymorphisms (AFLPs) (Tyrka et al., 2002, Lerceteau-Kohler et al., 2003) and inter-simple sequence repeats (ISSRs) (Debnath et al., 2008). Then came microsatellites (Folta et al., 2005, Bassil et al., 2006a, 2006b; Gil-Ariza et al., 2006; Keniry et al., 2006; Sargent et al., 2009; Vilanova et al., 2008; Rousseau-Gueutin et al., 2010; Zorrilla-Fontanesi et al., 2011a), which allowed the development of more saturated linkage maps (van Dijk et al., 2012; Isobe et al., 2013). By using their high level of transferability, comparative mapping within *Fragaria* (Rousseau-Gueutin et al., 2008; Sargent et al., 2009) was initiated. Results suggested a close relationship within *Fragaria* (Rousseau-Gueutin et al., 2008).
High-throughput genotyping based on single nucleotide polymorphisms (SNPs) is no longer hampered by the genetic complexity of the cultivated strawberry. Exploiting next generation (NGS) sequencing and use of a targeted capture technique to identify SNPs allowed the development of a saturated linkage map (Tennessen et al., 2013) and more in depth comparative genomics (Tennessen et al., 2014). By combining phylogenetic analysis and high throughput genotyping, Tennessen et al., (2014) showed numerous rearrangements in three out of the four subgenomes of *F.* ×*ananassa* because of local introgression of *F. vesca*. Furthermore, this study confirmed that one of the four subgenomes originated with the diploid cytoplasm donor *F. vesca* subsp. *bracteata,* another with the diploid *F. iinumae*, while the remaining two subgenomes appeared to originate from an unknown ancestor close to *F. iinumae*. Additional platforms for high-throughput SNP genotyping were recently developed and include DArT (Sanchez-Sevilla et al., 2015) and Affymetrix® Axiom ®arrays (Bassil, Davis et al., 2015). They were both used successfully for developing saturated octoploid linkage maps.

Unraveling the organisation of the cultivated strawberry genome

Using cytology, *Fragaria x ananassa* formulas that reflect genome structure were developed and elicited constant debate. In 1946, on the basis of meiosis observation by crossing the cultivated strawberry with different diploid species, Federova (1946) suggested AACCBBBB where A represents *F. vesca*, and other genomes were poorly defined. The presence of the four genomes B suggested a monophyletic original duplication. Thereafter, Senakaye and Bringhurst (1967) changed it to AAA'A'BBBB to reflect high homology between the A and C genomes. More recently, the formula became AAA'A'BBB'B' because of the behaviour of the genome as a diploid (Bringhurst 1990). In 2009, a study based on the phylogeny of two genes (Rousseau-Gueutin et al., 2009) supported the model proposed by Senakaye and Bringhurst (1967) by presenting *F. vesca* or an ancestor to be the origin of genomes A and A' and *F. iinumae* for genome B (formula AAA'A'BBBB). Recently, a phylogenomic approach using multiple well distributed single copy genic sequences (Tennessen et al., 2014) showed that only one of the four subgenomes originates with the diploid cytoplasm donor of *F. vesca*, one with *F. iinumae*, while the remaining two belong to an unknown ancestor close to *F. iinumae*. They propose a genomic formula of AABBB'B'B''B'', and name the subgenomes Av, Bi, B1 and B2. In addition, these authors found that extensive unidirectional introgression has converted *F. iinumae*-like subgenomes to be more F*. vesca*-like, but never the reverse. This last observation could explain the results of phylogenetic analyses that used sequences of two genes (Rousseau-Gueutin et al., 2009) since these regions could have been subjected to this type of introgression (Liston pers comm). In a subsequent study of genetic linkage mapping with the Axiom array, Sargent et al., (2016) obtained congruent results to Tennessen et al., (2014). To highlight the finding in both studies that the B1 and B2 subgenomes cannot be confidently assigned across homoeologues, they used the formula AA,bb,X-X,X-X. However, they conclude that X1 and X2 (equivalent to B1, B2) are practical to use for referencing individual chromosomes.

Correct segregation of chromosomes is especially demanding in polyploid species which contain more than two sets of chromosomes that need to be sorted out during meiosis to produce balanced gametes (Cifuentes et al., 2010). Formation of bivalents could be genetically controlled such as in wheat (Sidhu et al., 2008) or in rapeseed (Jenczewski et al., 2003). In strawberry, regular meiosis is usually observed (Byrne and Jelenkovic 1976). Analyses of coupling/repulsion phases suggest the prevalence of disomic behaviour in the cultivated strawberry, despite the possible existence of residual levels of polysomic segregation. These results are supported by the latest cytological formula, which includes three B genomes (Tennessen et al., 2014). This residual polysomic behaviour is likely to suggest that diploidization is currently an ongoing process in the cultivated strawberry.

Genotyping revealed several important characteristics of the cultivated strawberry genome such as residual homozygosity. This residual homozygosity was suggested (i) by the number of polymorphic alleles for SSRs (this was based on the hypothesis of 8 alleles by SSR; Rousseau-Gueutin et al., 2008) and (ii) by large genomic regions lacking polymorphic markers in a highly dense linkage map (Van Dijk et al., 2014). This residual homozygosity could be due to the limited number of genotypes at the origin of the cultivated strawberry. By tracing pedigrees of 134 North American cultivars, Dale and Sjulin (1990) suggested their origin from only 17 cytoplasmic sources.

What tools do we have to study the relationships between gene function and plant phenotype in strawberry?

With the development of high throughput technologies, large efforts have been devoted in diploid and octoploid strawberry to generate collections of Expressed Sequence Tags (ESTs) from various plant tissues (Bombarely et al., 2010; Folta et al., 2010; Kang et al., 2013; Hollender et al., 2014). This information allowed the construction of gene expression arrays used to monitor the expression of strawberry genes in a large variety of organs and conditions (Munoz blanco). As the cost of high throughput sequencing decreased, RNAseq emerged as an essential tool for studying gene expression (Sánchez-Sevilla et al., 2014; Chambers et al., 2014).

Gene expression data can be further combined with other genomics data, typically metabolome, to assign putative functions to the genes. Data could be examined using visualization tools such as Pathway-Tools platform (Naithani et al., 2016) and further analysed using various statistical means such as correlation network analysis (Pillet et al., 2015).
However, correlative information is by itself not sufficient to assign a function to a gene. Gene function and role in planta is usually inferred by the analysis of phenotypic alterations triggered by changes in transcript level or alteration of the gene under study (Rothan et al., 2016). Analysis of the function of a single gene or of few genes is classically done by stable genetic transformation of strawberry (diploid or octoploid) with *Agrobacterium* (diploid: El Mansouri et al., 1996; Oosumi et al., 2005; octoploid: Barceló et al., 1998) or by transient expression via agro-injection (Hoffmann et al., 2006) or Virus-Induced-Gene-Silencing (VIGS; Tian et al., 2014).

Studying the relationship between a gene polymorphism and the corresponding phenotypic variation can be performed using Nearly Isogenic Lines (NILs) (Urrutia et al., 2015). These lines are also excellent tools to dissect quantitative characters and identify some of their components as Mendelian traits rather than a direct relationship between gene and phenotype. The introgressed fragment in the line may carry tens of genes susceptible to affect the trait studied.

Germplasm resources represent a large source of wild and cultivated genetic variability for strawberry in which natural allelic variants underlying phenotypic changes can be found. As an example, a perpetual flowering diploid was one of these variants and the gene underlying this trait was the floral

repressor *TFL1* (Iwata et al., 2012; Koskela et al., 2012). Yellow fruit observed in 'Yellow Wonder' was due to a mutation in the *MYB10* gene (Hawkins et al., 2016; Zhang submitted).

Recently, Hytonen and co-workers also demonstrated the power of population genomic approach on the identification of naturally selected loci with potential roles in adaptation by using a large diploid collection consisting of accessions from across the European continent. They also initiated genome wide association mapping approach to screen the strawberry genome for significant associations between SNPs and specific phenotypic alterations such as flowering time (Toivainen, Hytonen, RGC8 Angers 2016).

On a larger scale, functional genomics can rely on the analysis of mutant collections, which have been developed or are currently being produced. After generating T-DNA or transposon-tagged lines (Oosumi et al., 2010; Veilleux et al., 2012), collections of EMS mutants appear to be very promising (Denoyes, Slovin pers comm).

Unravelling complex traits

A wide array of traits are targeted for genetic improvement in strawberry by plant breeders, and the choice of the trait varies from region to region depending on cultural management, producer needs, consumer demands, market preferences, and industrial requirements for better strawberries. Availability of the genome sequence enables clearer understanding of the genome architecture and can result in unravelling the basic mechanisms involved in complex traits. The genome sequence serves as the foundation for deploying genomics in crop improvement to accelerate the rate of genetic gains by identifying the loci/genes/alleles responsible for economically important traits.

To unravel complex traits by using genomics approaches and to develop a molecular strategy for breeding, mapping approaches were developed and include: (1) linkage mapping/quantitative trait locus (QTL) mapping and (2) association mapping/linkage disequilibrium (LD) mapping. Different types of populations with variability in the target traits are now available in strawberry: pseudo_F_1 (e.g. Lerceteau-Kohler et al., 2012; Zorrilla-Fontanesi et al., 2011b), F_2 (Monfort pers comm), and multi-parent mapping populations (nested association mapping) (Whitaker comm pers). This last population type has the advantages of both bi-parental (high power of QTL detection) and association mapping (high resolution).

Unravelling complex traits by using genomics approaches and developing a strategy for breeding will be shown through examples on flowering, fruit quality and disease resistances. Because of possible rearrangement of the genome between the octoploid and the diploid relative, markers developed for marker assisted selection have to be developed in the cultivated species, *F.* ×*ananassa*.

<u>Perpetual flowering / Everbearing / Day-neutrality</u>

Perpetual flowering is a major agronomical trait, because it permits the extension of the fruit production season. The *FaPFRU* locus linked positively to the extension of flowering and negatively to runnering (Gaston et al., 2013) resulted from an introgression of a perpetual flowering locus from a *F. virginiana* subsp. *glauca* Wasatch (Bringhurst and Voth, 1984; Powers, 1954). Several markers linked to this trait have been developed (Castro et al., 2015; Honjo et al., 2016). More recently, markers tightly linked to the locus and framing it were developed based on a selective mapping approach using a reduced sample of individuals (Perrotte et al., 2016). These markers are currently being used for marker assisted selection (Petit, Bassil, pers comm). These markers were all located on *F. vesca* subsp. *bracteata* pseudochromosome 4 (Perrotte et al., 2016 Supplemental Figure 1).

For the correct timing of flowering, the *FLOWERING LOCUS T1* (FvFT1) – SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (FvSOC1) – TERMINAL FLOWER1 (FvTFL1) pathway is essential

(Mouhu et al., 2013). Using transgenic plants for the seasonal flowering cultivar Elsanta, Koskela et al., (2016) showed that TFL1 is an essential target for breeding. Identifying alleles conferring new phenotypes should improve this trait.

Phenolic compounds

By using the *F. vesca* NIL collection (Urrutia et al., 2015), the genetic dissection of the metabolomites (metabolomic QTL (mQTL) responsible for the variability of metabolites) was performed (Urrutia et al., 2016). Because these lines can be grown easily, in addition to genotype effect, the environmental effect on the variation of metabolites can be studied. With comparative mapping based on the saturated maps thanks to the development of the Axiom array (Bassil, Davis et al., 2014), markers identified in these NILs could be tested in the cultivated strawberry.

Aroma

Strawberries are highly appreciated for their flavour, which results from a combination of sugars, acids and volatile organic compounds (VOCs). Despite the interest in flavour improvement, markers linked to this trait are difficult to develop due to the complexity of the different primary and secondary biochemical pathways, which in addition are regulated by developmental and environmental cues (klee et al., 2010). Despite these challenges, a comprehensive study was able to identify QTLs controlling 48 different VOCs during different seasons (Zorrilla-Fontanesi et al., 2012). This study highlighted a high stability of about 50% of the QTLs when grown in the same cultivation system during three consecutive years. This result is in contrast with other studies that have shown a wide environmental influence on VOCs concentrations in strawberry (Olbrich et al., 2011). The content of a reduced number of the studied VOCs, such as mesifurane and γ-decalactone, are controlled by major genes, enabling their identification (Zorrilla-Fontanesi et al., 2012). A combination of metabolomics and expression studies in the progeny lines of this mapping population resulted in the identification of *FaOMT* as the gene controlling natural variation in mesifurane content in strawberry (Zorrilla-Fontanesi et al., 2012). Later, two groups used complementary, yet different, approaches and segregating populations to identify the *FaFAD1* gene, required to synthesize γ-decalactone, which provides “peachy” notes in strawberry (Chambers et al., 2014; Sánchez-Sevilla et al., 2014). *FaFAD1* was shown to be essential, as lines with a deletion of this gene were not able to accumulate the VOC. Markers developed for these two important VOCs have been developed and validated in a diverse collection of strawberry cultivars and are available for genetic screening (Iraida Amaya manuscript in preparation).

Resistance to *Xanthomonas fragariae*

Angular leaf spot caused by *Xanthomonas fragariae* is a bacterial disease of cultivated strawberry in many countries. Wild accessions US4808 and US4809 were previously identified as resistant to the four genetic clades of *X. fragariae*, and introgression of the trait into commercial germplasm was initiated by Dr. Andrew Jamieson in Nova Scotia (Jamieson et al., 2014). Previous studies showed high heritability for the trait and both single-locus and multi-locus inheritance models (Lewers et al., 2003). To identify causal loci and introgress resistance into Florida-adapted germplasm, resistance was observed in two years of field trials with inoculated plants that assayed four full-sib families descended from US4808 and US4809. Resistance segregated 1:1 in all families indicating control by a dominant allele at a single locus. Using a selective genotyping approach with the IStraw90 Axiom® SNP array and pedigree-based QTL detection with FlexQTL™, a single QTL with large effect was identified in two full-sib families, one descended from each resistant accession. High-resolution melt curve analysis validated the presence of the QTL in separate populations. The QTL was delimited to the 33.1 - 33.6 Mbp (*F. vesca vesca* v1.1 reference) and 34.8 - 35.3 Mbp (*F. vesca bracteata* v2.0 reference) regions of linkage group 6D for both resistance sources and was designated *FaRXf1*.

What next?

Release of a reference genome of octoploid species will considerably improve the knowledge of gene function and genome functionality. The availability of these well-assembled genomes will allow us to study epigenetic (Gu et al., 2016) and other modifications that appeared during the process of polyploidization (from diploid to octoploid) and interspecific cross (between *F. virginiana* and *F. chiloensis*).

Breakthrough advances in the last few years including the strawberry genome sequencing (Shulaev et al., 2011), the availability of tens of thousands of genetic markers distributed over the whole genome (Bassil, Davis et al., 2015) will greatly facilitate the identification of causal alleles responsible for a particular phenotype. As a consequence, the map-based cloning process will not be the next bottleneck. The next bottleneck will be high throughput phenotyping. Techniques are already developed for evaluating high number of samples for their fruit quality. However, phenotyping will be more challenging for developmental processes such as floral induction/initiation.

With post genomics objectives, considerable efforts have to be devoted to perform thorough phenotypic analysis of natural diversity, mostly in the cultivated strawberry but also in its wild relatives, and to store these data in web accessible databases (e.g. GDR) that can be browsed in search of trait variations. The availability of these new phenotypic, genetic and genomic resources will result in greater accuracy and efficiency of marker selection for use in DNA-informed breeding.

Important areas such as proteomics approaches including proteome mapping and comparison of protein profiles for different genotypes/tissues in *Fragaria* should be developed in strawberry. These data will enhance our understanding of interaction networks through protein–protein interactions.

Finally, the integration of metabolomics, proteomics with transcriptomics datasets, high-throughput phenotyping and bioinformatics platforms, for profiling of large and genetically diverse populations, will enable the identification of novel metabolic QTLs and enhance the identification of candidate genes for traits of interest.

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